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13. ABSTRACT *(Maximum 200 Words)*

The enzyme, protein kinase C (PKC), is composed of a family of at least 11 isoforms which have distinct functions in signal transduction, tumor progression, and apoptosis. Elevated levels of PKC have been observed in breast cancers. The purpose of this project is to enhance radiation treatment through the use of isoform specific PKC inhibitors. Human breast tumor cell lines were treated with isoform specific AO (antisense oligonucleotides) and radiation. Cell survival and PKC isoform protein reduction were measured. Future experiments will address DNA damage following AO and radiation treatments as well compare the effectiveness of AO, chemical PKC inhibitors, and dominant negative PKCs in augmentation of radiation induced cell death. Results show that inhibition of PKC δ and ζ by AO in combination with radiation treatment significantly reduces cell survival in two distinct breast tumor cell lines, while inhibition of PKC α, ε, or η does not. Western blotting shows that AO are effective inhibitors of PKC isoforms, with PKC α and ζ protein levels markedly reduced by AO treatment. These results support the use of isotype specific PKC inhibitors in combination with radiation treatment as a potential cancer therapy.

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Meredith A. McCracken 7/20/2000

PI - Signature Date

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Introduction:

The purpose of this project is to augment radiation treatment by targeting PKC (protein kinase C), a serine/threonine kinase involved in signal transduction, tumor cell progression and apoptosis. There are at least 11 known isoforms of PKC classified into 3 sub-types: classical (α, β, and γ), novel (δ, ε, η, μ, and θ) and atypical (ζ, ι, and λ) (Glazer, 1998). Aberrant PKC signaling has been observed in breast cancer cell lines and we hypothesize that inhibition of particular PKC isoforms will increase the sensitivity of breast tumor cells to radiation treatment. Human breast tumor cell lines (MCF-7 and MDA-231) will be treated with a combination of radiation and PKC AO (antisense oligonucleotides) targeted to each of the PKC sub-types. Cell survival, protein reduction, and DNA damage assays will be performed following the PKC AO and radiation treatments. The goal of this project is to develop a cancer therapy by combining radiation and PKC isotype selective antitumor agents. First, through the use of PKC AO, PKC isoforms which when inhibited enhance radiation induced cell death will be identified. Following identification of these PKC isoforms, isoform selective drugs will be compared to PKC AO for efficiency in augmenting radiation treatment.

Body:

Research Accomplishments

The first objective of this project is to determine optimal conditions for the delivery of AO (antisense oligonucleotides) to cells. To optimize conditions, different lipid transfection reagents were tested for their ability to deliver a GFP (green fluorescent protein) plasmid to MCF-7 cells. DOTAP (*Boehringer Mannheim*) transfection reagent yielded 25% of MCF-7 cells which were positive for GFP expression, in comparison to a lower percentage of GFP positive cells using Lipofectin (*Gibco BRL*), Lipofectamine Plus (*Gibco BRL*), or electroporation. In addition DOTAP was used to deliver an FITC-labeled AO (Objective # 2) and resulted in 42% uptake of the FITC-AO. However, due to inherent differences between a plasmid or FITC-AO and the PKC AO, such as size, chemistry, and subcellular destination, is now clear that the uptake of plasmids and FITC-AO are not the best indicators for the efficiency of a lipid in AO delivery, because AO uptake is only the first step in the mechanism of action of an AO. Following uptake, AO must be released from the lipid carrier into the cytoplasm where they should bind their target mRNA with efficiency and specificity causing activation of RNAse H which catalyzes target mRNA degradation, resulting in inhibition of protein production. Based on this information, a protocol using Lipofectin with conditions for AO delivery optimized for maximal mRNA and protein reduction provided by Isis Pharmaceuticals was adopted. This transfection protocol is also consistent with the manufacturers guidelines. Co-transfection experiments (Objective # 2) with FITC-AO and subsequent flow cytometry to enrich for cells that have taken up the FITC-AO will not be performed, because uptake of the FITC-AO is not indicative of PKC AO uptake and target degradation. Objective # 1 is complete.

Objective # 3 of the research proposal is to quantify the reduction of PKC isoform mRNA levels by Northern analysis. This objective is modified in that Western blot analysis with PKC isoform specific antibodies is used to quantify protein reduction by PKC isoform specific AO. Protein levels are considered an appropriate end point versus mRNA levels since ultimately it is the reduction of PKC protein which is of interest. The P.I. spent the month of June, 1999 learning the Western blot technique and optimizing for antibodies to PKC isotypes. Partial completion of this objective showed a kinetic response to PKC α active AO with a 95% reduction in PKC α protein levels at 96 hours post-AO treatment compared to the control, a scrambled PKC α AO sequence, in MDA-231 cells (Figure 1). PKC α protein levels were not detectable in MCF-7 cells there for protein reduction could not be quantified. Future experiments using a different cellular protein extraction protocol which yields higher protein concentrations than the previously used extraction method will be used to quantify PKC α levels in MCF-7 cells. Treatment of MDA-231 cells with PKC ζ active AO produced a 73% decrease in PKC ζ protein levels at 24 hours post-AO treatment compared to the PKC ζ scrambled AO (Figure 2a). Treatment of MCF-7 cells with PKC ζ active AO produced a 70% reduction in PKC ζ protein levels at 48 hours post-AO treatment compared to the PKC ζ scrambled AO (Figure 2b).

Objective # 4 is to compare the sensitivity of breast tumor cells to radiation following treatment with either PKC AO or chemical PKC inhibitors. Cell survival following PKC inhibition and radiation treatments will be used in order to compare the sensitivity of cells to radiation. Cell survival assay optimization has been completed (Task # 5) so that relative survival of control versus treated cells may be quantified. The assay consists of plating dilute densities of cells in 96 well plates, allowing 5-7 days growth, and measuring the number of

viable cells by a MTS assay (*Promega*). The MTS assay is advantageous over the formerly used MTT assay in that the MTS reagent forms a readily soluble product when in the presence of viable cells, while the MTT assay requires a solubilazation step. The MTS assay in addition to a new microtiter plate reader (*Molecular Devices*) purchased by the P.I.'s department have allowed highly sensitive detection of cell survival in 96 well plates. The MTS assay allows faster acquisition of data than conventional crystal violet staining. Using the MTS assay, a panel of PKC AO (α, δ, ε, η, and ζ) have been screened to determine the PKC isoforms that are potential targets for anticancer drugs. To date all PKC AO supplied by Isis Pharmaceuticals have been screened in the MCF-7 and MDA-231 cell lines, completing objective # 2 with the exception of flow cytometry. Treatment of the two cell lines with PKC α, δ, ε, η, and ζ AO have produced similar results (Figure 3a and b). PKC δ and ζ AO treatments significantly reduced cell survival following 5.6Gy radiation compared to treatment with the scrambled AO and radiation in MCF-7 cells. PKC δ and ζ active AO also produced a significant decrease in the survival of MDA-231 cells following radiation treatment in comparison to the respective scrambled AO. Results show that of the five PKC isoforms tested, PKC δ and ζ are the most promising targets. There for approaches other than AO for targeting PKC δ and ζ will be tested such as chemical PKC inhibitors designed to be isoform specific, like Rottlerin to PKC δ (Way, 2000), and dominant negatives to PKC δ and ζ. In addition results from MTS assays will be further supported by the crystal violet staining assay. Comparisons in the effectiveness of PKC AO versus chemical PKC inhibitors will complete objective # 4.

Preliminary data suggest that the PKC δ and ζ signal transduction pathways do not act synergistically, as combinations of PKC δ and ζ AO treatments in MCF-7 or MDA-231 cells do not produce additive effects.

Objective # 5 intends to address DNA damage induced by radiation and PKC inhibitors. The proposal originally stated that pulse-field gel electrophoresis would be used to assess DNA damage. Since the time of the original proposal the comet assay has become widely used and published in the fields of radiation biology and oncology. The comet assay allows for detection of single or double strand DNA breaks at a single cell level. One advantage of this assay over pulse-field gel electrophoresis is that no radioactivity is required. Instead DNA is stained with a fluorescent dye, like propidium iodide, and visualized by fluorescence microscopy. In the future the P.I.'s laboratory will house a fluorescent microscope appropriate for use with the comet assay, which will offer increased convenience over use of the phosphorimager required for visualization of DNA in the pulse-field gel electrophoresis procedure. The basic principle of the comet assay is that lysed cells are run in an electrophoretic field with intact DNA confined to cell nuclei forming a "comet" while broken DNA migrates behind the comet as a "tail". Analysis of the tail will allow for determination of a threshold and a percentage of cells that have DNA damage above or below the threshold value may be determined.

Training Accomplishments

In addition to the above stated research accomplishments the P.I. has made several training achievements during the July 1, 1999 through June 30, 2000 funded year. Per the requirements of the P.I.'s Genetics and Developmental Biology Ph.D. Program, written and oral qualifying examinations were successfully completed in December of 1999. The P.I. attended the American

Society for Cell Biology Meeting held in Washington, DC in December, 1999. In the spring semester of 2000, the P.I. completed two required courses: Quantitative Genetics as well as Yeast and Mammalian Signal Transduction, while maintaining an overall 3.77 GPA. In April, 2000 the P.I. published and presented results from the funded research project at the American Association for Cancer Research meeting held in San Francisco, CA. At this meeting new and existing collaborations pertaining to the funded project were established and maintained.

Figure 1: PKCα Protein Expression in MDA-231 Cells

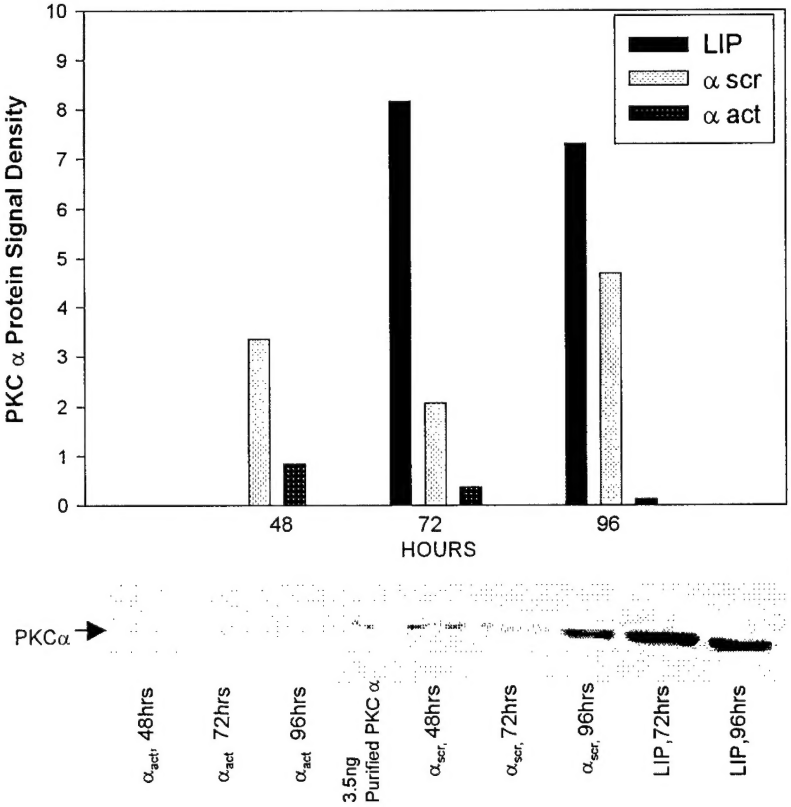

Figure 1: 6x10^5 MDA-231 cells were treated with 100nM PKC α active or scramble antisense oligonucleotide and Lipofectin:Opti-Mem transfection reagent (LIP) for 4 hours. 48-96 hours post-transfection total cellular proteins were isolated. 20ug of whole cell extract proteins were resolved on a 7.5% acrylamide gel and analyzed by Western blot. Data shown represent one experiment.

Figure 2a:
PKC ζ Protein Expression MDA-231 Cells

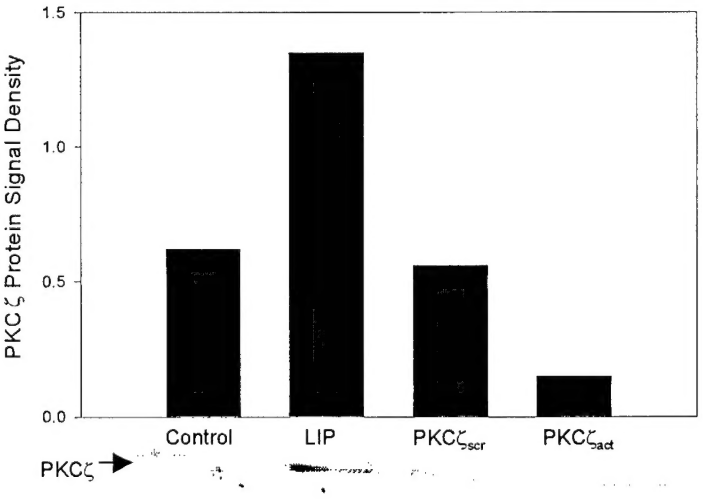

Figure 2a: 1.1x10^5 MDA-231 cells were treated with 50nM PKC ζ active or scramble antisense oligonucleotide and Lipofectin:Opti-Mem transfection reagent (LIP) for 4 hours. 24 hours post-transfection total cellular proteins were isolated. 55ug of whole cell extract proteins were resolved on a 7.5% acrylamide gel and analyzed by Western blot. Data shown represent one experiment.

Figure 2b:
PKC ζ Protein Expression in MCF-7 Cells

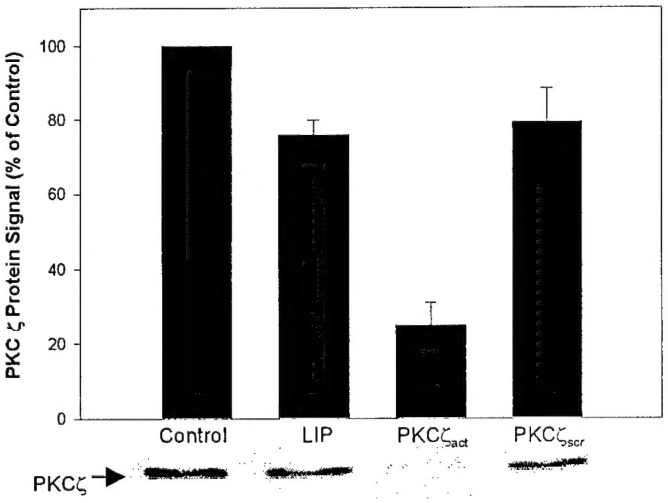

Figure 2b: 2.2x10^5 MCF-7 cells were treated with 100nM PKC ζ active or scramble antisense oligonucleotide and Lipofectin:Opti-Mem transfection reagent (LIP) for 5 hours. 48 hours post-transfection total cellular proteins were isolated. 55ug of whole cell extract proteins were resolved on a 7.5% acrylamide gel and analyzed by Western blot. Graph data represent the mean of 2 experiments and standard error is shown. Signal density from control cells was set =100%.

Figure 3a: MCF-7 Cell Survival Following PKC Antisense Oligonucleotide and Radiation Treatments

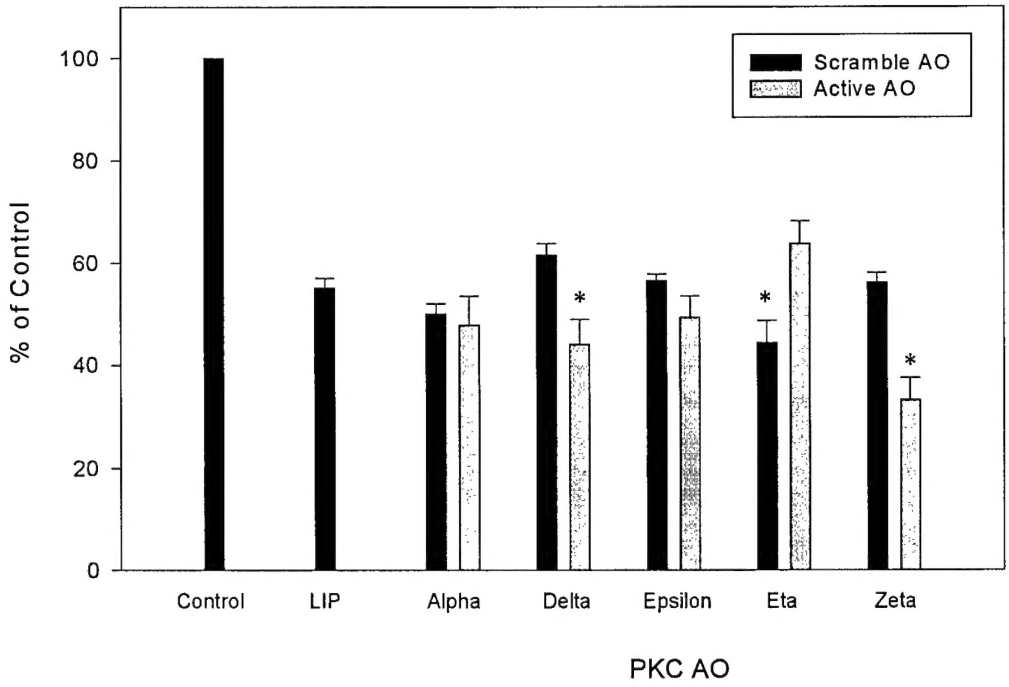

Figure 3a: $2.2x10^5$ MCF-7 cells were treated with 200nM PKC active or scrambled antisense oligonucleotide by Lipofectin:Opti-Mem transfection reagent (LIP) for 5 hours. 48 hours post-transfection cells were irradiated with 5.6Gy, harvested, and replaated at cloning cell densities. Cells were grown for 7 days and cell survival determined by MTS assay. Data represent mean from 3 experiments with 5 replicates per treatment per experiment. Standard error is shown. Cell survival of irradiated cells (Control) was set =100%.
* Indicates statistical significance between active and scrambled PKC antisense oligonucleotides by unpaired t-test.

Figure 3b: MDA-231 Cell Survival Following PKC Antisense Oligonucleotide and Radiation Treatments

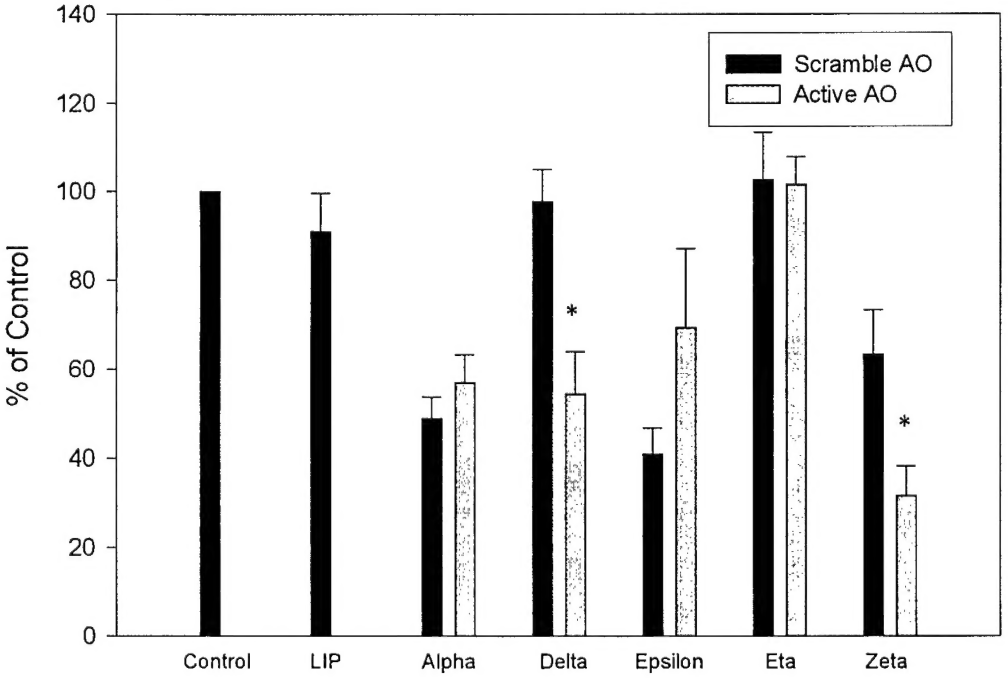

Figure 3b: $1.1x10^5$ MDA-231 cells were treated with 100nM PKC active or scrambled antisense oligonucleotide by Lipofectin:Opti-Mem transfection reagent (LIP) for 5 hours. 24 hours post-transfection cells were irradiated with 5.6Gy, harvested, and replated at cloning cell densities. Cells were grown for 5 days and cell survival determined by MTS assay. Data represent mean from 4 experiments with 5 replicates per treatment per experiment. Standard error is shown. Cell survival of irradiated cells (Control) was set =100%.
* Indicates statistical significance between active and scrambled PKC antisense oligonucleotides by unpaired t-test.

Key Research Accomplishments:

1. Conditions for delivery of AO optimized. (Objective # 1)

2. Optimization of cell survival assay- MTS (Task # 5).

3. Western blot: PKC α and ζ protein reduction by PKC AO (Objective # 3).

4. PKC AO tested for ability to increase sensitivity of MCF-7 and MDA-231 cells to radiation treatments. Experiments were repeated at least 3 times. (Objective # 2 and # 4).

Reportable Outcomes:

- Abstract and Poster Presentation- American Association for Cancer Research Meeting, April 1-5, 2000, San Francisco, CA
  "The Effects of Protein Kinase C Antisense Oligonucleotides on Mammary Tumor Cell Lines." M.A. McCracken, L. Miraglia, and J.S. Strobl, Abstract # 4491 Proceedings of 91st Annual American Association for Cancer Research, Vol. 41.

Conclusions:

In effort to enhance the sensitivity of breast tumor cell lines to radiation treatment, isotype specific PKC inhibitors have been studied. Antisense oligonucleotides specific to PKC isoforms, α, δ, ε, η, and ζ, have been used to treat MCF-7 and MDA-231 cell lines. The first obstacle in treatment of the cells with PKC AO is AO delivery. Cationic lipid transfection reagents were chosen to deliver the AO and Lipofectin was found to be the optimal lipid reagent. Treatment of MCF-7 and MDA-231 cell lines with PKC AO and Lipofectin results in marked reductions the respective PKC isoform protein levels. In addition, treatment of MCF-7 and MDA-231 cell lines with PKC δ or ζ AO combined with radiation treatment reduces the survival of the cells compared to treatment with radiation alone or radiation combined with control PKC AO sequences. A cell survival assay (MTS), which provides quick data acquisition and sensitive detection of viable cells has been optimized. In the future the effectiveness of PKC AO in augmenting radiation induced cell death will be compared to chemical PKC inhibitors and PKC dominant negatives. Experiments to address DNA damage induced by radiation and PKC inhibition will be performed. Finally, normal mammary cell lines will be treated with radiation and PKC inhibitors.

References:

Glazer, R.I. (1998) The Protein Kinase ABC's of Signal Transduction as Targets for Drug Development. *Current Pharmaceutical Design* 4:277-290.

Way, K.J., Chou, E., and King, G.L. (2000) Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends in Pharmacological Science* 21:181-187.

Cited Abstracts:

American Association for Cancer Research Meeting, April 1-5, San Francisco, CA,

Abstract # 4491

"The Effects of Protein Kinase C Antisense Oligonucleotides on Mammary Tumor Cell Lines."
M.A. McCracken, L. Miraglia, and J.S. Strobl

A positive correlation exists between PKC α (protein kinase C) levels and breast tumor aggressiveness. PKC ζ acts in the activation pathway of the anti-apoptotic transcription factor, NFκB. In an effort to increase the sensitivity of human mammary carcinoma cells to IR (γ-ionizing radiation), overexpressed PKC isotypes are targeted with AO (antisense oligonucleotides). PKC protein isoform profiles showed MDA-231 cells have high levels of PKC α, while MCF-7 cells display low PKC α and high PKC ζ levels. We tested the effects of PKC α and ζ phosphorothioate AO on the radiosensitivity of MDA-231 and MCF-7 cell lines. PKC α AO (50nM) decreased PKC α protein by 87% in MDA-231 cells compared to cells treated with AO_{scr} (scrambled). PKC α AO caused a 3-fold decrease in clonogenic survival following 4.4Gy IR compared to AO_{scr}. PKC ζ AO (100nM) decreased PKC ζ protein by 74% in MCF-7 cells compared to AO_{scr}. MCF-7 cells showed a 1.6-fold decrease in clonogenic survival following 5.6Gy IR compared to AO_{scr}. Isoform specific PKC AO show promise as radiosensitizers of human breast tumor cells.

Supported by USAMRMC Grant #BC980899 and Susan G. Komen Foundation, Inc.

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